CONTRACT NO.: DAMD17-90-Z-0043

TITLE: MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXINS TETANUS TOXIN

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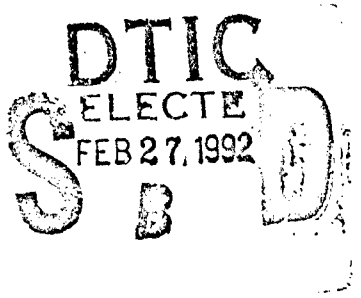

REPORT DATE: January 25, 1992

TYPE OF REPORT: Midterm Report

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK
FREDERICK, MARYLAND 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 25, 1992	Midterm 1 Jul 90 - 30 Dec 91

4. TITLE AND SUBTITLE
Mechanism of Action of the Presynaptic Neurotoxin Tetanus Toxin

5. FUNDING NUMBERS
DAMD17-90-Z-0043
62787A
3M162787A871 AA
DA335463

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

During the past year a number of enzymatic systems have been investigated as possible targets for the action of the Clostridial neurotoxins. A cultured cell system, PC12 cells, was used as a model to exam specific systems. We have examined the effects of tetanus toxin on protein kinase C levels in such cells and have found that there is no significant inhibition during intoxication. Further, we have initiated studies to look at the effects of botulinum and tetanus toxins on cyclicnucleotide phosphdiesterase systems. We have fractioned the PDE activity by ion exchange chromatography and have found that there are multiple forms in PC12 cells and that treatment with nerve growth factor causes a marked shift or change in the pattern of PDE expression along with the growth factor induced differentiation. These studies provide the basis for future studies that will examine the effects of toxins on these specific enzyme subtypes.

14. SUBJECT TERMS
Clostridial neurotoxins; RA 1; Botulinum toxin; Secretion; Clostridial infection; Neurons; Neurotransmitter release

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TB Rogers 1/25/97

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TABLE OF CONTENTS

TABLES

FIGURES

INTRODUCTION

Tetanus toxin, the enterotoxin produced by the bacterium *Clostridium tetani*, is one of the most potent neurotoxins known (minimal lethal dose of toxin in mice, 2 ng/kg body weight). This toxin shares many common properties with botulinum toxin, a group of neurotoxic substances also produced by Clostridial bacteria. These toxins have a common bacterial origin, similar molecular structures, and most likely the same mechanism of toxic action at the subcellular level (for recent reviews see Simpson, 1990; Habermann and Dreyer, 1986). The most striking feature in the action of these toxins, beside their potency, is that their site of action is the presynaptic nerve terminal where they inhibit neurosecretion without causing cell death. Thus studies on the mechanism of action of the Clostridial neurotoxins should not only provide methods to prevent or reverse the toxic sequelae of these lethal bacterial infections but will also provide valuable insight into the molecular events that underlie the neurosecretion process.

It has been recognized for some time that the effects of tetanus toxin are specific for neural tissues, which is due, in part, to the specific recognition of neural tissues by the toxin. Evidence gathered by the principal investigator and others supported the notion that the specific high affinity receptors for tetanus toxin were polysialo-gangliosides (Rogers and Snyder, 1981; Walton *et al.*1988; Staub *et al.*1986). However, there has also been evidence to suggest that protein plays some role in the high affinity binding site (Pierce *et al.*1986; Critchley *et al.*1986). Thus the precise nature of the tetanus toxin receptor remains to be characterized and more work is needed to assess the physiological importance of gangliosides as binding molecules.

It is now clear that the initial binding step of the Clostridial toxins is nontoxic. In fact tetanus is like several other microbial toxins that participate in a complex multi-step intoxication pathway (Middlebrook and Dorland, 1984). Various steps in the pathway have been studied in

neural tissues. (Schmitt *et al.*1981; Bergey *et al.*1983; Collingridge *et al.*1980). Recently, the principal investigator, utilizing an established preparation of tetanus toxin-sensitive PC12 cells, clearly identified a rapid, temperature-dependent internalization step following toxin binding to the surface (Sandberg *et al.*1989). Further, there was a clear lag phase which followed internalization, revealing that other intracellular events, such as processing of the toxin and expression of some enzymatic activity, are obligatory events in the pathway (Sandberg *et al.*1989). At present there is no information on the toxin processing events, the compartments in which they occur, or on the enzymatic activity or substrates of tetanus toxin. The PC12 cell system developed by the Principal Investigator represents an ideal system in which to address these important issues.

An emphasis of recent research has been to identify the putative enzymatic activity of the Clostridial neurotoxins. By analogy with other toxins, such as diphtheria and cholera, a number of investigations have focused on a potential ADP-ribosyltransferase activity for tetanus and botulinum. Although certain forms of botulinum toxin (C1 and D) can ADP-ribosylate a low molecular weight protein in adrenal medulla, there is evidence that this reaction is not related to inhibition of neurosecretion (Adam-Vizi *et al.*1989). In the case of tetanus toxin, there is no evidence for ADP-ribosyltransferase or any other enzymatic activity for that matter (Simpson, 1986). The lack of information on the precise target or substrate for these toxins has made progress difficult in this area. One of the important goals of this proposal is to identify these substrates and develop probes so that the underlying enzymatic activity of tetanus toxin can be discovered.

The molecular mechanism that underlies the inhibitory effects of the Clostridial neurotoxins are not known. There is some evidence that the Ca^{2+} sensitivity of the release process is decreased (Mellanby and Green, 1981). Although there is one report that tetanus toxin blocks Ca^{2+} channels in cultured neuronal cells, substantial evidence indicates that neither tetanus nor

botulinum toxin act on Ca^{2+} channels (Dreyer *et al.*1983; Simpson, 1986). cGMP was implicated in the toxic action with the report that Clostridial neurotoxins inhibited guanylate cyclase in neural tissues (Smith and Middlebrook 1985). The principal investigator has obtained substantial evidence to implicate cGMP metabolism with the action of tetanus toxin in PC12 cells (Sandberg *et al.*1989). There has also been an interesting recent report that tetanus toxin decreases protein kinase C activity in macrophages and neural tissues from infected mice (Ho and Klempner, 1988). Consistent with this result are recent reports in which protein kinase C stimulated secretion in permeabilized pituitary cells and PC12 cells (Naor *et al.*1989; Ahnert-Hilger and Gratzl, 1988). The precise relation between these different observations is unclear One of the important goals of this research project is to identify an underlying relationship, or lack thereof, between protein kinase C, cGMP and tetanus toxin in neurosecretion.

RESULTS

The experimental approaches that have been pursued during the first half of this research grant have been closely organized around the "Statement of Work" that was submitted with the original proposal. The overall strategy has been to utilize a model system of cultured cells of neural origin, originally established by the Principal Investigator (Sandberg *et al.*1989), to examine the molecular mechanisms of action of the Clostridial neurotoxins. The focus of our efforts has been to examine the effects of Clostridial toxins of enzymatic systems in the PC12 cells that may play crucial roles in neurosecretion. Although some of the results have been negative, a great deal of progress has been made toward an understanding of the cGMP/phosphodiesterase system in NGF-treated PC12 cells. Thus these preliminary results place us in an excellent position to examine the role of these enzymes in Clostridial neurotoxin action.

Experimental Series 1 -- Do Clostridial neurotoxins alter protein kinase C activity in cultured neuronal cells ? In this phase of the research plan we have examined the hypothesis that the action of Clostridial neurotoxins is causally related to a decrease in protein kinase C (PKC) activity. This part of the project has been stimulated by the growing awareness that PKC is involved in secretion in a variety of cells (Naor *et al.*1989; Ahnert-Hilger and Gratzl, 1988), and from recent observations that tetanus toxin reduces PKC activity in neural tissues of intoxicated mice (Ho and Klempner, 1988).

PC12 cells were cultured on multiwell dishes in the presence of NGF for 8-10 days by methods previously described by the PI (Sandberg *et al.*1989; Walton *et al.*1988). The cells were then be incubated for 16 hr with tetanus toxin in concentrations from 10 nM to 1 μM. Following these incubations, the cells were homogenized and the cytosol and particulate fractions were

separated. PKC was solubilized from the membrane fraction with Nonident NP-40 and then resolved by DEAE ion exchange chromatography. These methods have been previously described (Thomas *et al.*1987). The PKC activity was then be measured in extracts of the soluble and particulate fractions of cell homogenates by previously described methods (Thomas *et al.*1987; Alloatti *et al.*1990; Lai *et al.*1990; Doerner *et al.*1990). The PKC activity was assessed by the ability of the fractions to stimulate the phosphorylation of histone (Type III) *in vitro* and were calculated as the Ca^{2+}-phospholipid stimulated nmol [^{32}P]PO_4 incorporated /min/mg protein. The results are shown in Table 1.

TABLE 1

Effects of Tetanus Toxin on Protein Kinase C activity in PC12 Cells

Culture Conditions	PKC Activity (nmol ^{32}P/min/mg protein) Cytosol	Particulate
SPARSE	1.4 ± 0.2	0.3 ± 0.08
+NGF	2.4 ± 0.15	0.8 ± 0.18
+NGF, + Tetanus Tox.	2.1 ± 0.18	0.9 ± 0.20

As shown in Table 1, when PC12 cells were incubated with 100 nM tetanus toxin for 16 hr, there was no effect of the toxin on the steady state levels of PKC in the cultures. There was no change in the distribution of the enzyme between the soluble and particulate fractions, and the specific activities were nearly identical under the two incubation conditions. It is important to note that we have previously demonstrated that such tetanus toxin incubations result in 80% inhibition of neurotransmitter release in PC12 cells. Thus, these results argue against an important role for

PKC in the Clostridial toxin intoxication process.

These results are not consistent with previous studies in which intrathecal injection of tetanus toxin into spinal cord of mouse resulted in a significant decrease in the levels of PKC in this multicellular tissue (Ho and Klempner, 1988). There are a number of potential reasons for the discrepancy, certainly not the least of which is the difference in the systems used. Thus, tetanus toxin infections may lower PKC activity in non-neuronal cells in the preparation. Such events would not be detected in the homogeneous population of neuronal cells in PC12 cultures.

Experimental Series 2 -- Do Clostridial Neurotoxins Modify cyclic nucleotide phosphodiesterase activities in whole cell homogenates from PC12 cells? Previous work from our laboratory has suggested that Clostridial neurotoxins act by increasing cGMP phosphodiesterase (PDE) activity in neural cells (Sandberg *et al.*1989; Sandberg *et al.*1989). Thus initial studies were performed to see if increases in cGMP PDE activity could be observed in whole cell homogenates of tetanus toxin-treated PC12 cells. PDE activity was determined in the homogenates using a combined two-step procedure as described previously (Kincaid and Manganiello, 1988). The reaction was initiated by addition of the enzyme preparation to an incubation mixture containing, in a final volume of 300 µl, 10nM [^{3}H]cAMP or [^{3}H]cGMP, 1 µM cAMP or cGMP, 1mM MgCl, 0.1mM EGTA, 0.2% soybean trypsin inhibitor, and 0.2mg/ml BSA in 50mM BES buffer, pH7.4. The hydrolysis of cAMP or cGMP catalyzed by PDE was usually allowed to proceed for 60 min at 30°C. Following the termination of the hydrolytic reaction 5'-nucleotidase from snake venom was used to convert 5'-nucleotide product derived from cAMP or cGMP hydrolysis to the corresponding nucleoside. The conversion was complete for 10-20 min at 30°C. The final products, [^{3}H]adenosine or [^{3}H]guanosine, were separated from the unreacted substrate by ion exchange chromatography using DEAE-Sephadex A25. As shown in Figure 1, significant levels of Mg^{2+}-

dependent cGMP PDE activity was observed in whole cell extracts from NGF-treated PC12 cells.

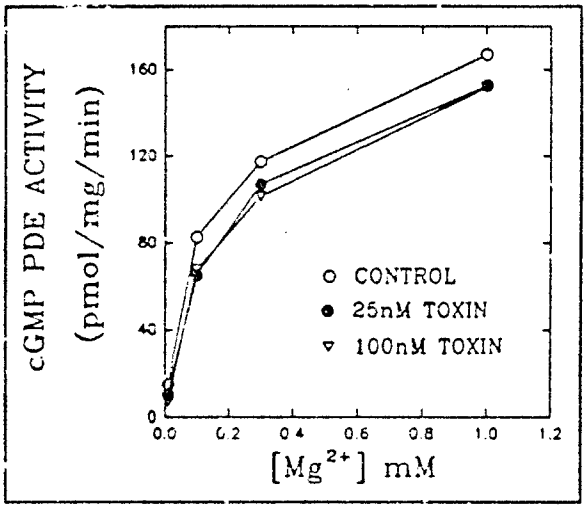

Figure 1. Effects of tetanus toxin on cGMP PDE activity in PC12 cells. NGF-treated cells were exposed to 100 nM tetanus toxin overnight. Whole cell extracts were prepared from control (○), toxin-treated (25 nM (●) or 100 nM (▽)) cells. The cGMP PDE activity as a function of Mg^{2+} concentration was determined.

Figure 1 also shows that tetanus toxin pretreatment has no effect on the resulting PDE activity in whole cell homogenates. However, it is now clear that the PDE activity in cells is a composite of many potential isoforms, each with distinct requirements for ions, such as Ca^{2+}, and other factors, such as calmodulin. Thus, the effects of tetanus toxin on cGMP PDE activities measured under different incubation conditions was assessed to determine if tetanus toxin was altering activity of one specific subtype of PDE.

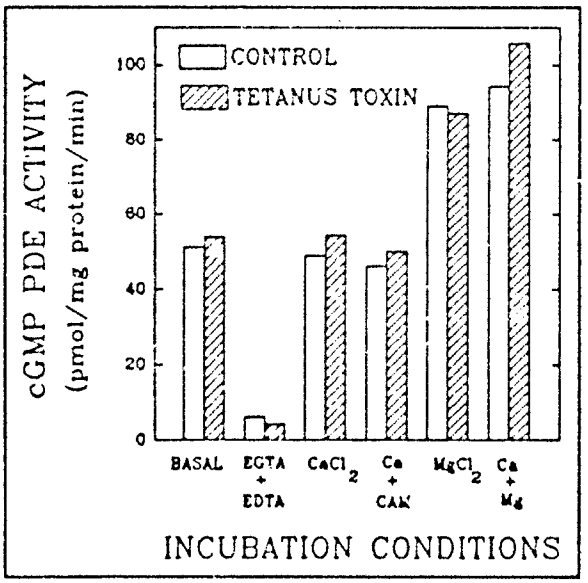

Figure 2. Effects of tetanus toxin on cGMP PDE activity. PC12 cells were incubated with 100 nM tetanus toxin overnight. Extracts were prepared and were assayed for cGMP PDE activity under the conditions shown. The conditions were; EGTA+EDTA (1 mM); $CaCl_2$ (50 μM); $CaCl_2$ (50 μM), Calmodulin (20 nM), MgCl, $MgCl_2$ (2mM). The results are reported as the percent activity, compared to extracts from control, non-toxin treated cells.

As shown if Figure 2, there were no detectable effects of tetanus toxin on whole cell extracts from PC12 cells under a variety of different ionic conditions. Thus, we failed to detect any effects of tetanus toxin on cyclic

nucleotide PDE activity in whole cell homogenates. However, it is still possible that Clostridial neurotoxins alter PDE activity but that it could not be observed under the experimental conditions used. For example, the activation of PDE activity could be reversed during the time required for preparation and assay of homogenates. Further, it is clear that there are multiple forms of PDE in any cell, thus tetanus toxin might be altering the activity of one specific isoform. Such activation may go undetected in the whole cell homogenate assay. Accordingly, other experiments were performed in order to explore these hypotheses in detail.

Experimental Series 3 -- Isolation and characterization of isoforms of cyclic nucleotide phosphodiesterase activity from PC12 cells. In order to understand cGMP metabolism in neural cells and the effects of Clostridial toxins on this system a detailed understanding of properties of the PDE isoforms present in PC12 cells is essential. Therefore in this experimental series, PDE isoforms were resolved from extracts of PC12 cells using ion exchange chromatography. PC12 cells were removed from culture dishes by incubating cells is in a dissociation buffer (Ca^{2+}- and Mg^{2+}-free phosphate buffer consisting of 137mM NaCl, 5.2mM KCl, 1.7mM Na2HPO4, 0.22mM EGTA, pH6.5, OSM340) for 5-10 min. The cells were collected by centrifugation and homogenized in 40mM Tris-HCl, pH8.0, containing 5mM $MgCl_2$ and 0.25mg/ml BSA The homogenate was subsequently used as a whole cell homogenate preparation for the PDE assays or was separate into soluble and particulate fractions by centrifugation. The PDE isoforms were resolved in the cytosolic fraction using ion exchange chromatography methods adapted from those previously described by Dicou et al. (1982) and Bode et al. (1988,1989). In brief, the soluble fraction (10-12 mg of protein) was loaded onto a DEAE-cellulose DE52 column (bed volume of 15 ml) which was previously equilibrated with 20mM Tris-HCl, pH7.4. The column was washed with two bed volumes of 20mM Tris-HCl, 2mM $MgCl_2$, pH7.4. PDE activity was eluted from the column with a linear gradient of 50-500mM NaCl in the same wash buffer. Fractions (1.5ml) were collected and

stored at -80°C. The eluting PDE activity was assayed as described above. Pilot experiments revealed that the PDE activity in these fractions was stable for at least 1 month at -70°C.

Initial experiments in this series focused on resolving major PDE species from undifferentiated and NGF-treated PC12 cell cultures. Cells were grown in flasks and the cytosol prepared as described above. About 10-12 mg of cytosolic protein was applied to the DEAE columns and the PDE activity measured in the eluting fractions. The results from these studies are shown in Figure 3.

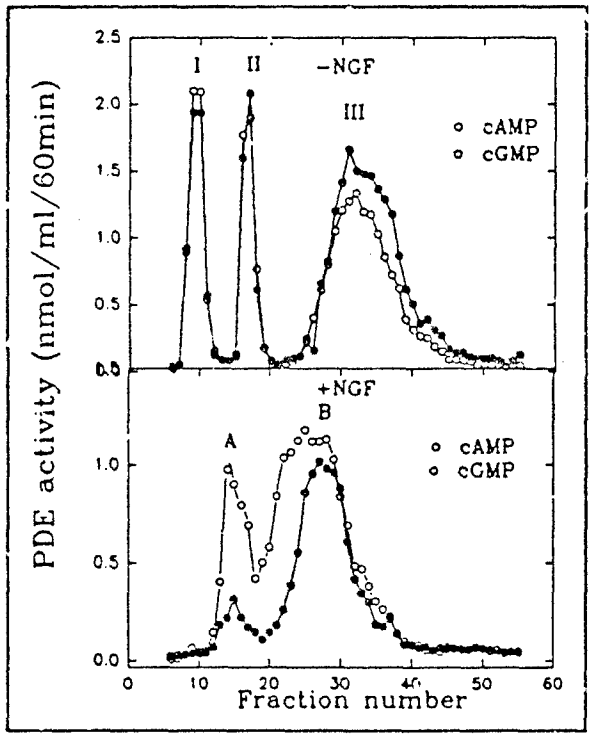

Figure 3. Chromatographic separation of PDE isoforms from PC12 cell extracts. PC12 cells were grown in the presence (Panel B) or absence (Panel A) of NGF. Cytosolic protein (10-12 mg protein) was resolved on DEAE cellulose columns as described above. Each fraction (1.5 ml) was subsequently assayed for cAMP- and cGMP-PDE activity.

Ion exchange chromatographic methods resolved three peaks of PDE activity from the non-NGF-treated cells (Figure 3A). The peaks were designated I, II, and III, in the order of their elution by the NaCl gradient. The hydrolytic activities of these fractions toward 1 μM cAMP or 1 μM cGMP as substrates were determined in all fractions. PDE activity in the three peaks exhibited no preference for either nucleotide. Figure 3B shows the chromatogram of PDE activity obtained from fractionation of cytosol obtained from NGF-treated cells. It was clear that there is a substantial difference in the profile of PDE activity in this differentiated system. The major differences can be summarized as follows. (1) Only two peaks, labelled A and B according

to the order of elution from DEAE-cellulose column, rather than three peaks seen in Figure 3A, were resolved from the NGF-treated cells. (2) The positions of two peaks were shifted so that neither peaks could precisely coincide with any peak appearing with the non-differentiated PDE preparations. These chromatographic profiles were reproduced in three different preparations with identical results (3) The PDE activity in Peak A appears to be very different from Peaks I and II in that the activity in Peak A showed a preference for cAMP as a substrate under the conditions used. Thus it is possible to resolve the cyclic nucleotide PDE activity of PC12 cells into multiple distinct species by ion exchange chromatography. Taken together, these results suppport the idea that NGF treatment causes a significant change in the expression of PDE species in PC12 cells.

The chromatographic results indicate that PC12 cells express distinctly different forms of PDE when cultured in the presence of NGF. This hypothesis was explored in more detail by the use of selective phosphodiesterase inhibitors. It is well recognized that different PDE isoforms display different sensitivities to synthetic inhibitors (Weishaar *et al.*1985). There is considerable controversy over the precise selectivity of synthetic inhibitors of PDE isoforms isolated from diverse sources. Yet, the demonstration of the inhibitory potencies of selective inhibitors of PDE activity has formed part of the criteria by which isoenzymes from different sources are characterized and classified. Accordingly, we examined the susceptibilities of all peaks of PDE activity, isolated as shown in Figure 3, to a variety of isozyme-selective inhibitors. The dose-inhibition curves are displayed in Figures 4 and 5.

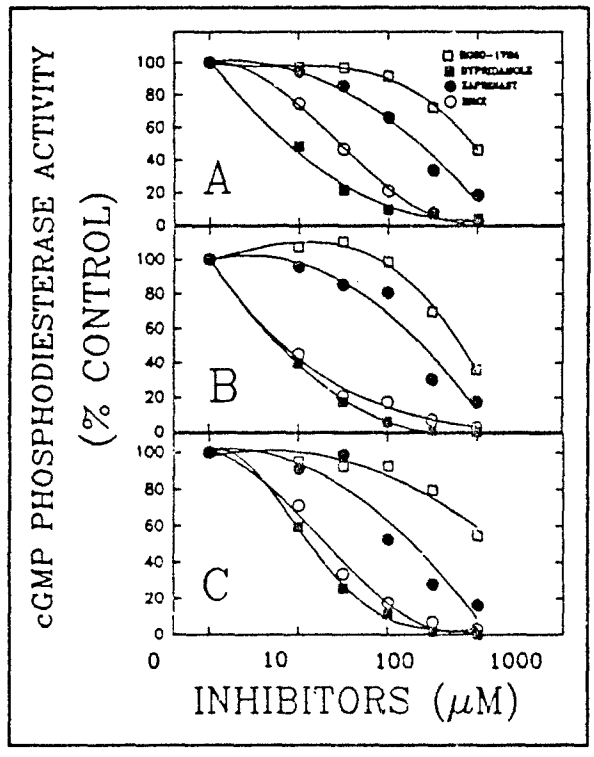

Figure 4. Effects of PDE inhibitors on PDE activity from nondifferentiated PC12 cells. The inhibitors used are displayed in the legend. The dose inhibition curves for PDE activity in peaks I, II, and III (Figure 3) are shown in Panels A, B, and C respectively.

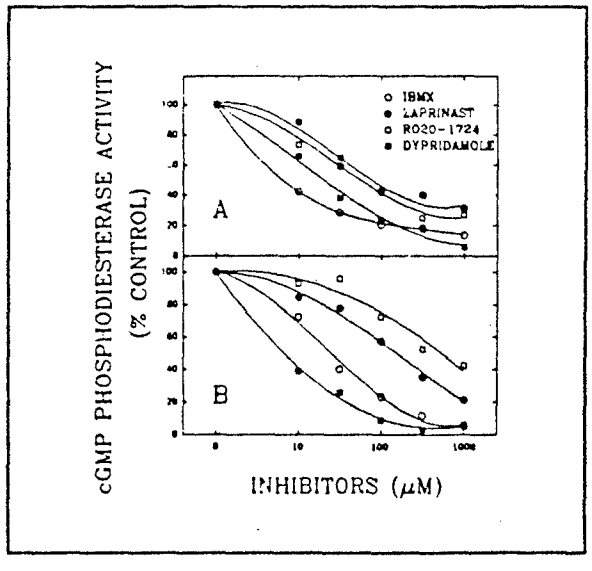

Figure 5. Effects of PDE inhibitors on PDE activity from NGF-differentiated PC12 cells. The inhibitors used are displayed in the legend. The dose inhibition curves for PDE activity in peaks A and B (Figure 3) are shown in Panels A and B, respectively.

PDE activity was measured by assessing the hydrolytic activity with 1 μM [^{3}H]cGMP as the substrate. The inhibition data from peaks I, II, and II from non-NGF-treated cells are plotted in

Figure 4 and the data from peaks A and B from NGF-treated cells are displayed in Figure 5. In general the PDE activities of all peaks could be inhibited by these PDE inhibitors in a dose-dependent manner. However, as shown in Figures 4 and 5, there were a number of differences in the inhibitory effects of the four selected inhibitors. In the non-NGF-treated cells, the rank order of inhibitory potency was identical in the three peaks; that is, dypridamole > IBMX > zaprinast > Ro20-1724. The first two inhibitors were much more potent, with IC_{50}'s in the range of 5-20 μM range and zaprinast in the 100-300 μM range. These data are summarized in Table 1. As shown in Figure 5, the pattern of inhibition in NGF-treated cells was clearly different. The rank order of potency for peaks A and B were IBMX > dypridamole > zaprinast > Ro20-1724 and dypridamole > IBMX > zaprinast >> Ro20-1724, respectively. Zaprinast was considerably more potent in inhibiting the NGF-cell PDE isoforms compared to those from non-differentiated cells. These data are summarized in Table 2 below.

TABLE 2

Sensitivity of PDE fractions to selective inhibitors

Inhibitor	IC_{50} (μM) -NGF Cultures			IC_{50} (μM) +NGF Cultures	
	I	II	III	A	B
IBMX	20	8	18	7	23
DYPRIDAMOLE	9	5	14	18	7
ZAPRINAST	222	300	100	50	60
Ro20-1724	800	600	1000	60	500

The four PDE inhibitors selected for the present experiments have a range of specificities.

IBMX is used widely as a non-selective inhibitor, whereas zaprinast, dypridamole, and Ro20-1724 are classified as selective inhibitors of PDE Typel, Type II, and Type III, respectively. Recent data supports the view that dypridamole is a PDE Type II selective inhibitor, it is also reported as a potent PDE Type V inhibitor. The fact that dypridamole exerted potent inhibitory effect on all isozymes from both cells with or without NGF treatment suggests that PDE Type II, cGMP-stimulated form of PDE (cGMPs-PDE), and PDE Type V, a cGMP-binding form of PDE (cG-BPDE), are probably the major isoenzymes expressed in PC12 cells.

A recent report has documented the presence of Type II PDE in PC12 cells (Whalin *et al.*1991). A common characteristic of this form of PDE is that it has a cGMP-stimulated cAMP PDE activity. A series of experiments were performed in order to examine which of the PDE peaks may be related to the type II isoform. PDE activity was resolved from NGF-treated extracts by ion exchange chromatography, as described above, and the cGMP-stimulated cAMP PDE activity was measured in each fraction. The results are shown in Figure 6.

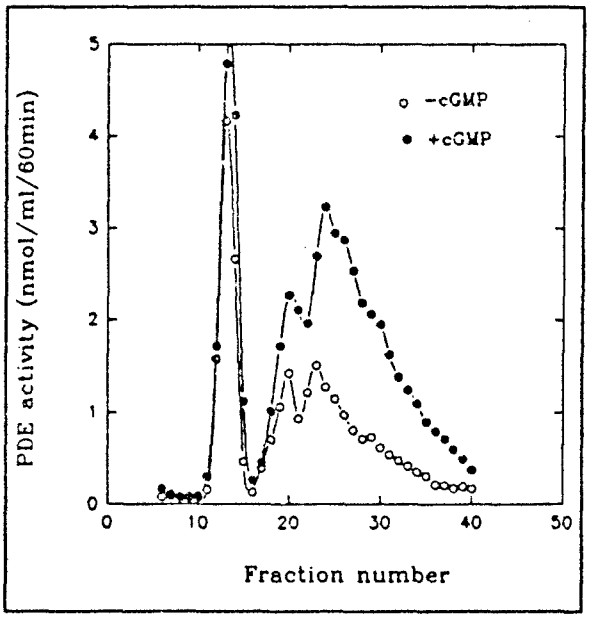

Figure 6 cGMP-stimulated PDE activity in fractions from NGF-treated PC12 cells. Extracts from PC12 cells were resolved on DEAE cellulose columns and the resulting PDE activity (toward 1 mM [^{3}H]cAMP) was determined in the presence or absence of 10 μM cGMP as indicated.

Although the PDE specific activity in Peak A was much larger, the activity was only minimally stimulated by cGMP. The region of Peak B has been resolved into two peaks as shown in Figure 6, with both being stimulated approximately

two-fold by cGMP. Double reciprocal plots from these peaks revealed that the main effect of 1 μM cGMP was to increase the V_{max} of the cAMP PDE activity from 9 to 24 μM min^{-1}, with little effect on the K_m for cAMP, 14 μM. These results are consistent with the typical Type II PDE activity regulation by cGMP.

Another property of specific PDE isoforms is their ability to bind cGMP. Thus cGMP binding assays were performed in order to further distinguish and characterize PDE isoforms in PC12 cells. cGMP binding activity in isolated PDE fractions was measured in a total volume of 250 μl in a buffer of 10 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 0.5 mg/ml histone IIA and 0.2 μM [^{3}H]cGMP, pH7.4 in the presence of 0.1 mM IBMX. The reaction was started by addition of the enzyme preparation and processed for 60 min at 4°C. Assay mixtures were then filtered onto Millipore HA filters (pore size, 0.45uM). The reaction tubes were rinsed with 4 ml of a 10mM Na_2HPO_4, pH7.4 and the filters were washed with 20ml of the same buffer. The radioactivities of the filters were counted in 5ml of scintillant. Nonspecific binding was estimated by performing the incubation without tissue or with tissue in the same assay mixtures at time zero. The specific binding activity was defined as the total amount of [^{3}H]cGMP bound minus the nonspecific binding component. The conditions employed for our binding assay were essentially derived from those described by Hamet et al. (1987) and Francis et al. (1988).

The cGMP binding activity of was determined in all of the PDE fractions and the results compared with cAMP PDE activity profiles. The results are shown in Figure 7.

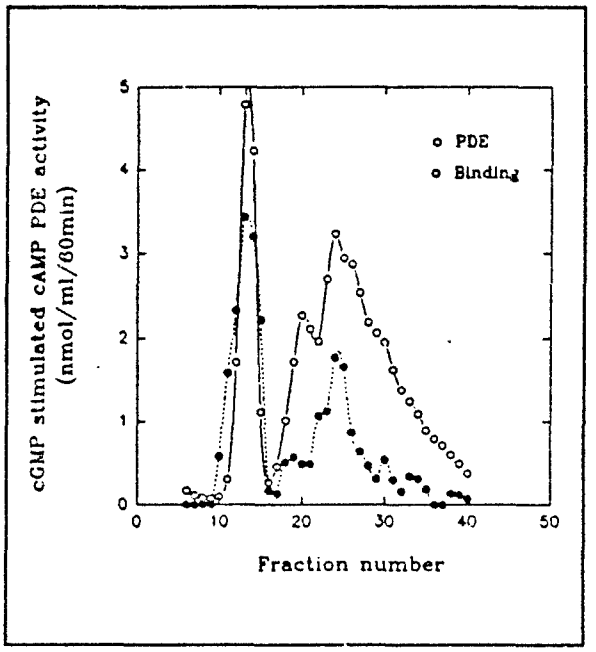

Figure 7. (file SPDEB)[^{3}H]cGMP binding activity of PDE fractions. Extracts from NGF-treated PC12 cells were resolved by ion exchange chromatography. Each fraction was assayed for cGMP-stimulated cAMP PDE activity (○) as well as for [^{3}H]cGMP binding activity (●)as described above. The binding activity was reported as fmol [^{3}H]cGMP bound/ml of solution. The results are reported for each fraction from the column.

As shown in Figure 7 there is significant [^{3}H]cGMP binding activity associated with the two major peaks of PDE activity, Peaks A and B. Peak B, which had a significant level of cGMP-stimulated PDE activity bound cGMP to a level of 200 fmol/ml. This is consistent with its designation as a Type II isoform. It is also clear that this large peak of activity is likely comprised of several distinct forms since there are areas of PDE activity that do not bind significant cGMP. Peak A, which did not show significant cGMP-stimulated PDE activity, did bind significant levels of cGMP, up to 400 fmol/ml. Thus it is not likely to be a Type II isoform, but may be related to the Type V isoform as recently reported. The sensitivity of this fraction to PDE inhibitors (Figure 3 and Table 2) is consistent with this view (Weishaar *et al.*1985).

Taken together, the data to date demonstrate that PC12 cells express multiple isoforms of PDE, each with distinct biochemical properties. An important discovery during this work is the observation that the expression of isoforms is highly dependent upon the differentiation state of the cells. Thus, culturing of PC12 cells with NGF results in a pattern of PDE expression that is very different from that seen with non-differentiated cultures. These differences were identified by changes in the mobility of PDE activity in ion exchange chromatography as well as by their differential sensitivities to selective PDE inhibitors. PDE activities were also distinct in their ability

to be stimulated by cGMP and by their cGMP binding properties. Thus by many criteria, it is demonstrated that NGF treatment results in the expression of a different group of PDE isoforms.

From our previous studies we have hypothesized that Clostridial neurotoxins act by altering the activity of a zaprinast-sensitive PDE isoform in neural cells (Sandberg *et al.*1989). Thus the data reported here are consistent with this with these previous studies in that there is a differential sensitivity of PC12 cells to tetanus toxin as a function of the differentiation state. Secretion in NGF-treated cells is sensitive to intoxication while neurotransmitter release in nondifferentiated cells is not sensitive to toxin treatment. Thus if PDE is a target for the toxins, then differential expression is a possible mechanism that underlies these results. Thus, a major goal in the near future will be to determine if any of the PDE isoforms that we have identified is modified by treatment with botulinum and tetanus toxins.

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